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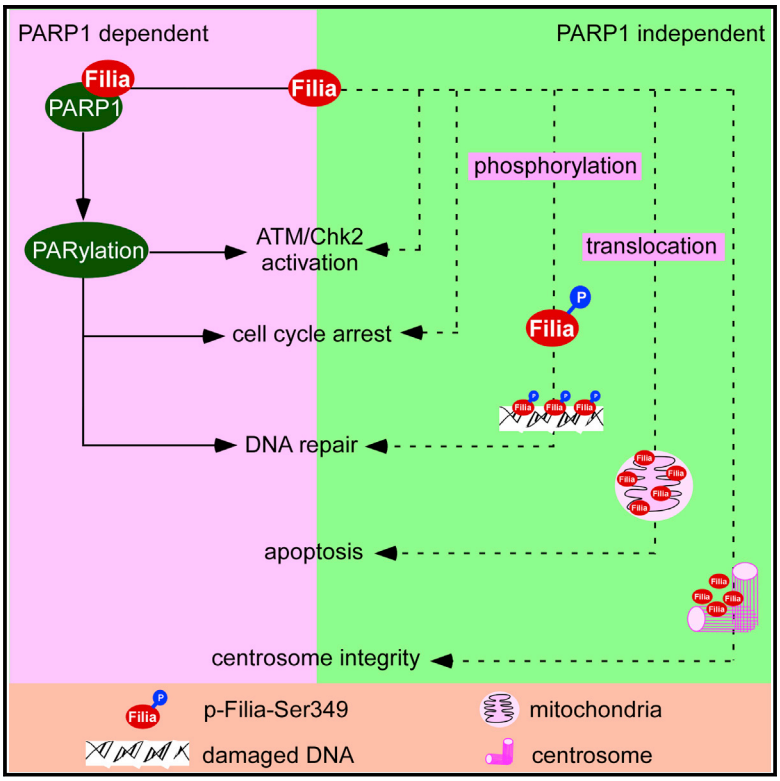
Filia Is an ESC-Specific Regulator of DNA Damage Response and Safeguards Genomic Stability

Graphical Abstract



Highlights

- Loss of Filia promotes genomic instability and transformation in ESCs
- Filia is induced by genotoxic stress and regulates repair responses
- Filia is regulated by post-translational modification and sub-cellular translocation
- Filia interacts with PARP1 and stimulates its enzymatic activity

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In Brief

Zhao et al. identify Filia as an ESC-specific regulator of genomic stability that is induced by genotoxic stress. Functional characterization highlights dynamic subcellular translocation and roles in governing multiple DNA damage response pathways, including stimulation of PARP1 enzymatic activity.

Zhao et al., 2015, Cell Stem Cell *16*, 684–698
June 4, 2015
http://dx.doi.org/10.1016/j.stem.2015.03.017

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Filia Is an ESC-Specific Regulator of DNA Damage Response and Safeguards Genomic Stability

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http://dx.doi.org/10.1016/j.stem.2015.03.017

SUMMARY

Pluripotent stem cells (PSCs) hold great promise in cell-based therapy, but the genomic instability seen in culture hampers their full application. A greater understanding of the factors that regulate genomic stability in PSCs could help address this issue. Here we describe the identification of Filia as a specific regulator of genomic stability in mouse embryonic stem cells (ESCs). Filia expression is induced by genotoxic stress. Filia promotes centrosome integrity and regulates the DNA damage response (DDR) through multiple pathways, including DDR signaling, cell-cycle checkpoints and damage repair, ESC differentiation, and apoptosis. Filia depletion causes ESC genomic instability, induces resistance to apoptosis, and promotes malignant transformation. As part of its role in DDR, Filia interacts with PARP1 and stimulates its enzymatic activity. Filia also constitutively resides on centrosomes and translocates to DNA damage sites and mitochondria, consistent with its multifaceted roles in regulating centrosome integrity, damage repair, and apoptosis.

INTRODUCTION

Pluripotent stem cells (PSCs) hold great potential for cell-based regenerative medicine. However, genomic instability and tumorigenicity limit their full applications. Understanding the mechanisms that regulate their genome stability is critical to address this issue. These mechanistic insights are also important to understand how pluripotent cells (e.g., germ cells and early embryos) sustain their genome integrity to ensure the successful development of an organism. Pluripotent cells are capable of developing into all cell types, whereas somatic cells are cell fate-restricted. Accordingly, pluripotent cells possess a higher competence than somatic cells to protect their genetic integrity.

DNA damage response (DDR) is a fundamental and evolutionarily conserved mechanism to preserve the genomic integrity of cells (Behrens et al., 2014; Jackson and Bartek, 2009). Upon DNA damage triggered by endogenous or exogenous insults, cells elicit complicated and highly coordinated response networks, including damage sensing and signal transduction, which trigger cell-cycle arrest and DNA repair. When the extent of DNA damage is beyond repairable, cells undergo apoptosis or senescence to prevent the passage of the mutations to descendent cell populations. These responses are coordinated at multiple levels of gene regulation, including at the transcriptional, post-transcriptional, translational, and post-translational levels. Recent advances have further extended our understanding of the DDR by documenting cytoplasmic Golgi dispersal as a novel component of the DDR network (Farber-Katz et al., 2014). Because of the importance of DDR in genomic stability, its dysfunction is closely associated with genetic diseases, tumorigenicity, and tissue aging (Bartkova et al., 2005; Liang et al., 2009; Rass et al., 2007). DDR has been studied intensively in somatic cells, and many key players have been identified. Compared with somatic cells, very few studies have been conducted in pluripotent cells regarding their DDR network components. Limited reports have suggested that PSCs employed distinct strategies to cope with DNA damage (Wyles et al., 2014). For instance, mouse embryonic stem cells (ESCs) bypass the G1/S cell-cycle checkpoint because of an extremely short G1 phase (van der Laan et al., 2013). Instead, intra-S and G2 cell-cycle checkpoints are critical for ESCs (Momčilović et al., 2011). PSCs predominantly employ error-free homolog recombination (HR) rather than the error-prone non-homologous end joining (NHEJ) pathway to repair DNA double-strand break (DSB) (Tichy et al., 2010). Moreover, PSCs utilize high mitochondrial priming and retention of constitutively active Bax at the Golgi to sensitize them to DNA damage (Dumitru et al., 2012; Liu et al., 2013). Although it is appreciated that DDR regulation in PSCs is distinct from that in somatic cells, the key players and their functional mechanisms remain unknown. In particular, PSC-specific DDR factors have never been identified.

Filia (official name, KH domain containing 3; also known as *Ecat1*) was first identified in mouse embryonic stem cells

(mESCs) (Mitsui et al., 2003). Its expression is restricted to undifferentiated ESCs. Later studies reported its expression in growing oocytes and identified two transcriptional isoforms. The long isoform (~1.6 kb) encodes a ~70 kDa protein and is predominantly expressed in ESCs, while the short isoform (~1.2 kb), encoding a ~50 kDa protein, is primarily detected in growing oocytes (Ohsugi et al., 2008). Functional analysis revealed that *Filia* is not essential for ESC self-renewal (Mitsui et al., 2003), whereas depletion of maternal Filia protein in oocytes led to severe aneuploidy in cleavage stage embryos (Zheng and Dean, 2009). Here we report Filia acts as a mESC-specific regulator of DDR and safeguards genomic stability.

RESULTS

Loss of Filia Causes Genomic Instability and Promotes Malignant Transformation of mESCs

To investigate the role of Filia in regulating genomic stability of mESCs, we derived three *Filia*$^{-/-}$ ESC lines—FK(I), FK(II), and FK(III)—and two wild-type (WT) counterparts from *Filia*-targeted mutant mice (Zheng and Dean, 2009). The success rates of ESC derivation did not differ between mutant and WT blastocysts (33.3%, [2 of 6] in WT versus 25% [3 of 12] in the *Filia* mutant), indicating that Filia is not required for the derivation of ESCs. Consistent with previous studies (Mitsui et al., 2003), loss of *Filia* did not impair the self-renewal of ESCs. FK ESCs displayed a comparable morphology, expression of pluripotency markers, alkaline phosphatase staining, formation of embryonic bodies, cell-cycle profiles, and growth competition ability compared with WT cells (Figures S1A–S1C). No overt morphological abnormality was observed in FK ESCs after 100 passages.

However, cytogenetic analysis of chromosome metaphase spreads revealed that FK ESCs exhibited severe chromosome abnormalities, including chromosome breaks (Figures 1A and 1B), fusion of chromosome ends (Figures 1A, 1C, and 1D), and sister chromatid exchange (SCE) (Figure 1E). Consistently, FK ESCs displayed a higher rate of chromosomal anaphase bridges (Figures 1B and 1F) and an increase in DNA damage markers such as γH2AX accumulation and focus formation (Figures 1G and 1H). The increase in DNA damage in FK ESCs was further validated by comet assay, a method that measures the extent of DNA damage on a single-cell basis (Figure 1I). Moreover, FK ESCs had high incidences of abnormal centrosomes, spindle assembly (Figure 1J), and aneuploidy (Figures S1D and S1E). These phenotypes were reproducibly observed in another ESC line with a distinct genetic background in which *Filia* expression was knocked down by an inducible short hairpin RNA (shRNA) (Figures S2A–S2E), indicating they are not genetic background-specific.

Genomic instability is known to promote cell transformation and tumorigenesis. Accordingly, we assessed the tumorigenicity of FK ESCs by injecting the unlabeled FK and GFP-labeled WT ESCs into the right or left testis of the same non-obese diabetic (NOD)/severe combined immunodeficiency (SCID) mouse. Teratomas formed by FK ESCs (GFP^{-}) weighed more than those formed by WT ESCs (GFP^{+}) (Figures 1K–1M). Furthermore, aggressive tumors lacking GFP expression were detected in the pancreas (Figure 1N), suggesting that they were formed by FK ESCs. Consistently, FK ESCs showed a delay in differentiation (Figure S1F). Therefore, we conclude that loss of Filia causes genomic instability and promotes tumorigenesis.

Filia Is Induced by Genotoxic Insults and Is Required for Activation of ATM and Chk2

Because FK ESCs displayed a severe DNA damage phenotype under normal culture conditions, we sought to determine whether Filia regulates DDR. Toward this goal, we investigated whether Filia expression is regulated by genotoxic insults. Indeed, the 70-kDa Filia was upregulated by DNA-damaging agents such as UV light, etoposide, doxorubicin, camptothecin, and hydroxyl urea (Nagy and Soutoglou, 2009; Figure 2A). The 50-kDa isoform was not detected in ESCs (Figure S3A). The expression and upregulation of Filia was specific to ESCs; mouse embryonic fibroblasts (MEFs) and mesenchymal stem cells (MSCs) did not express Filia in either the untreated or treated condition (Figure S3B).

We then systematically investigated the effects of Filia loss on major DDR signaling components, including γH2AX, ATM, ATR, Chk1, Chk2, and p53. Upon etoposide treatment, γH2AX, p-ATM, p-ATR, p-Chk1, and p-Chk2 were induced and sustained for at least 12 hr in WT ESCs (Figure 2B). In contrast, γH2AX and p-ATM were initially induced at comparable levels in FK and WT ESCs but failed to sustain in FK cells (Figure 2B; Figure S3C). Strikingly, Chk2, one of the key substrates of ATM, was not phosphorylated at all in FK ESCs (Figure 2B). This suggests that Filia participates in the Chk2 activation via mechanisms independent of ATM. Unlike the ATM-Chk2 signaling axis, ATR and Chk1 kinases were not significantly affected by Filia loss (Figure 2B; Figure S3D). ATM/Chk2 regulates p53 activity. Consistently, phosphorylation of p53 at its S15 and S20 was impaired in FK ESCs compared with WT ESCs (Figure 2B). To further define the domain necessary for regulating the signal transduction, we stably expressed full-length Filia, a Filia fragment containing amino acids (aas) 1–340, or hnRNP K homology (KH) domain (atypical RNA-binding domain) containing aas 1–125, in FK ESCs (FK-Filia, FK-340, and FK-KH rescue cells, respectively) (Figures S3A, S3E, and S3F). The induction of γH2AX, p-ATM, p-Chk2, and p-p53 were restored in FK-Filia and FK-340 (Figure 2B) but not in FK-KH cells (Figure S3G). These data revealed that the C terminus of Filia (aas 341–440) was dispensable for DDR signaling. Moreover, these functions were independent of the genetic background based on shRNA knockdown ESCs, as described above (Figure S2A).

In somatic cells, ATM and Chk2 activation exhibited pulsatile dynamics in response to DNA damage. Recurrent initiation of ATM/Chk2 activation is an important mechanism to sustain DDR (Batchelor et al., 2008). To better understand the dynamics of ATM/Chk2 activation in ESCs and the influence of Filia loss on ATM and Chk2 activation, we conducted a detailed time course analysis. WT ESCs displayed two waves of ATM activation. Filia loss did not affect the initial ATM activation between 1–4 hr post-damage but completely abolished ATM activity thereafter (Figure 2C). This suggests that distinct mechanisms regulate two phases of ATM activation, with the second phase relying on Filia. Unlike ATM, Chk2 activation did not exhibit discrete phases in ESCs. Moreover, Filia loss completely blocked Chk2 activation (Figure 2C), implying that Filia is necessary for Chk2 activation.

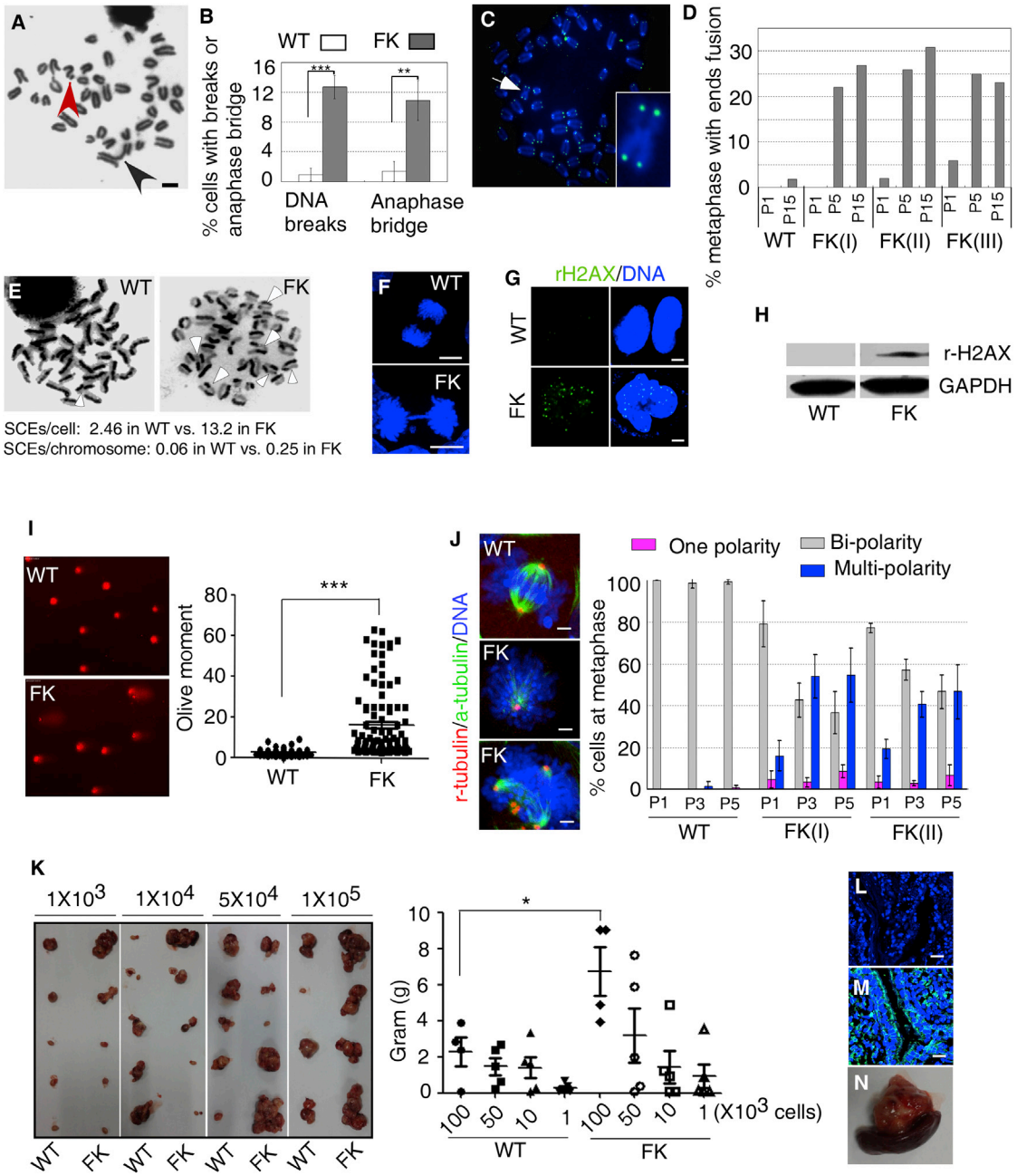

Figure 1. Filia Maintains Genomic Stability and Prevents Malignant Transformation of ESCs

(A) Metaphase chromosome spread of FK ESCs. Arrowheads indicate chromosome breaks (red) and chromosome end fusion (black).

(B) Frequencies of chromosome breaks and anaphase bridges in WT and FK ESCs. More than 200 cells were examined in each sample.

(C) Chromosome end fusion detected by T-FISH.

(D) Frequencies of metaphase with chromosome end fusion in WT and FK ESCs. More than 100 metaphase spreads were examined in each sample. P, passage.

(E) SCE (arrowheads) in WT and FK ESCs. 50 cells were examined in each group.

(F) Anaphase bridges in FK ESCs.

(G) γH2AX foci in WT and FK ESCs.

(H) γH2AX accumulation in WT and FK cells detected by immunoblotting.

(I) Comet assay showing that FK ESCs had severe DNA damage.

(J) Centrosomes in FK and WT ESCs at P3 and P5. More than 200 cells were examined in each of the indicated groups.

(K) Teratomas formed from FK ESCs were bigger and weighed more than those from WT ESCs injected at different concentrations.

(L) Teratoma tissue formed by FK ESCs that were GFP-negative.

(M) Teratoma tissue formed by GFP-labeled WT ESCs.

(N) FK ESC formed tumors in the pancreas.

Data are represented as mean ± SEM. Scale bars, 10 μm. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. See also Figures S1 and S2.

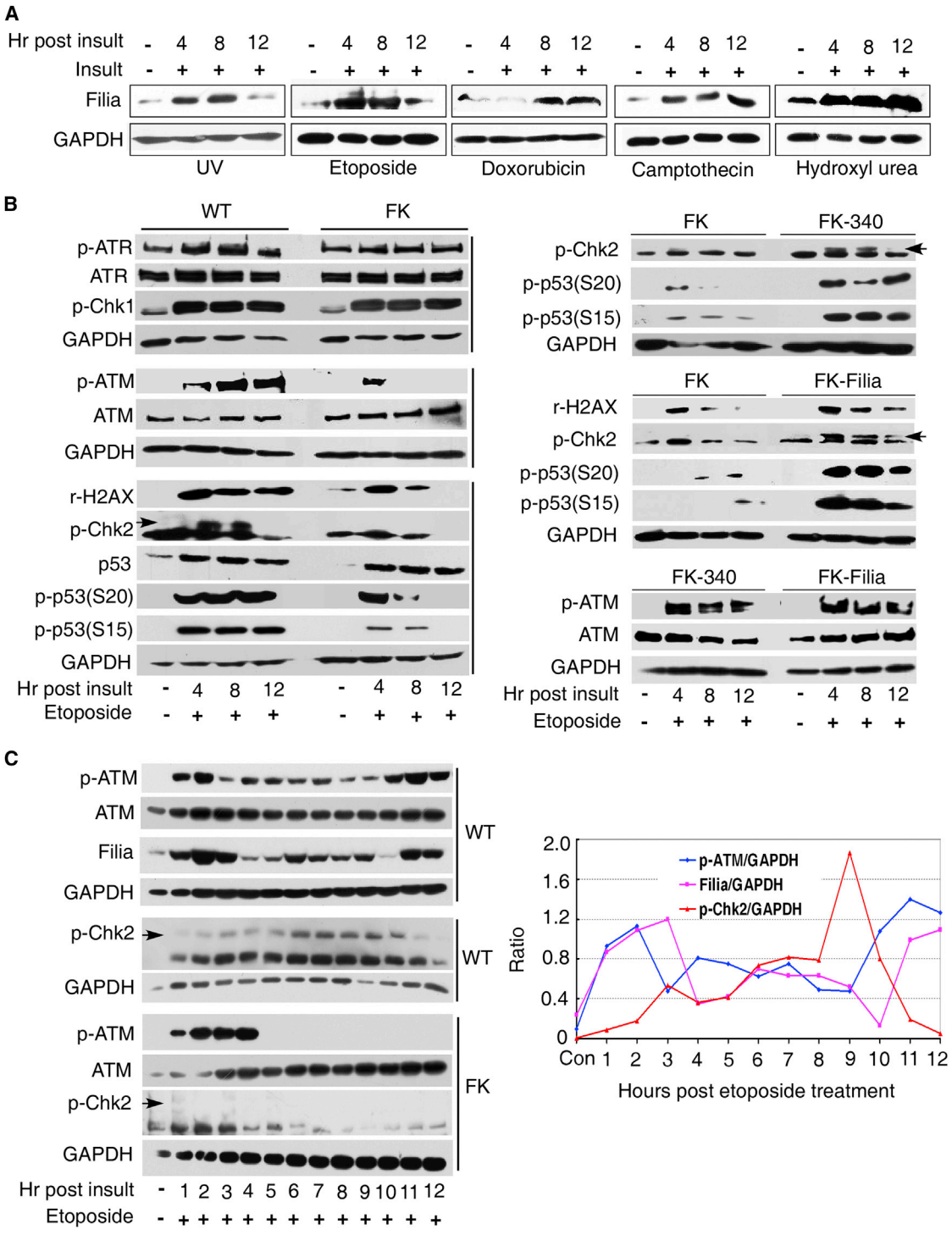

Figure 2. Filia Regulates DNA Damage Signaling

(A) Filia protein expression was upregulated by genotoxic insults.

(B) The induction of γH2AX, p-ATM, p-Chk2, and p-p53 was compromised in FK ESCs, whereas p-ATR or p-Chk1 was not affected. Re-expression of Filia or Filia340 successfully rescued the defects.

(C) Detailed time course analysis of p-ATM, p-Chk2, and Filia expression in WT and FK ESCs treated with etoposide. Arrows indicate p-Chk2.

See also Figures S2 and S3.

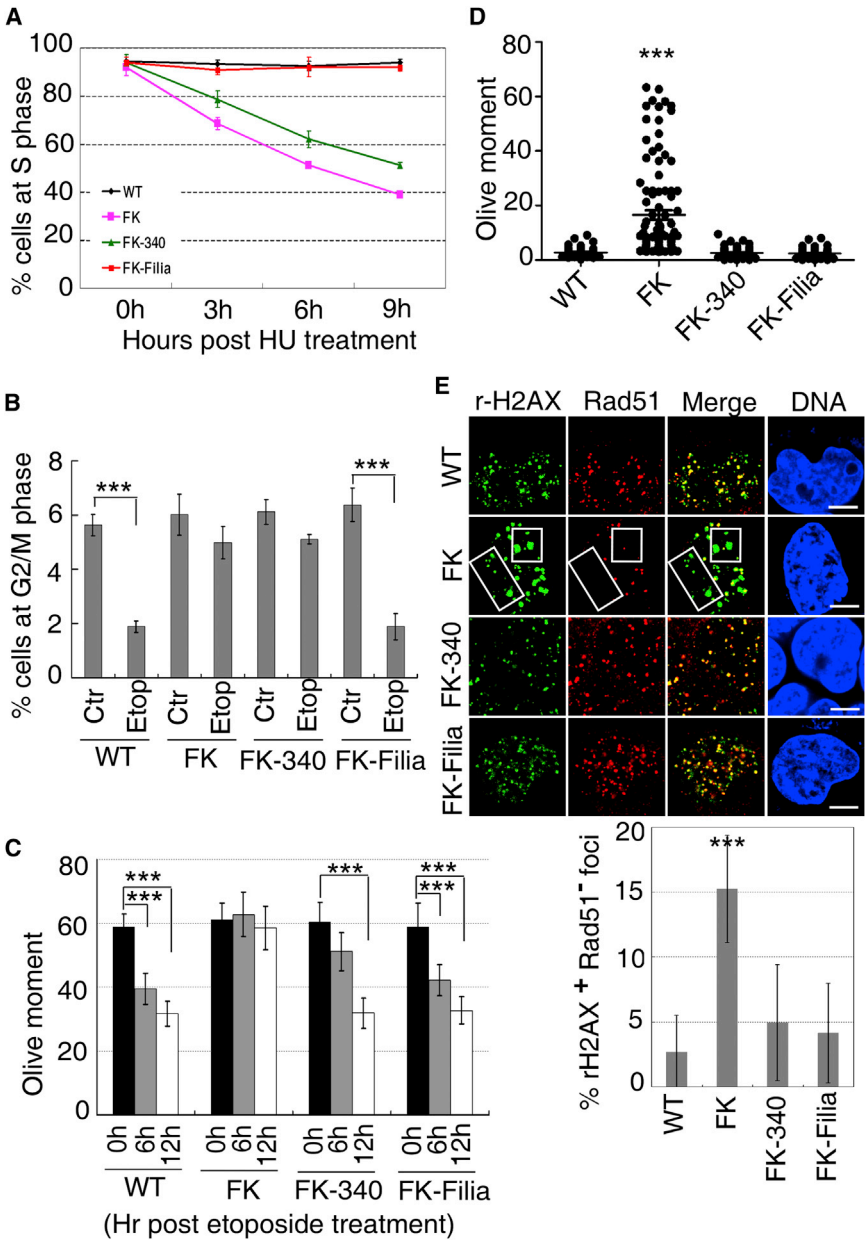

Figure 3. Filia Is Necessary for Cell Cycle Checkpoints and DNA Damage Repair

(A) The S phase checkpoint was functional in WT and FK-Filia rescue ESCs but failed in FK and FK-340 cells. HU, hydroxyl urea.

(B) Similarly, FK ESCs lost the G2 checkpoint, which was restored in FK-Filia but not FK-340 ESCs. Ctr, control. Etop, etoposide.

(C) WT, FK-Filia, and FK-340 ESCs could repair DNA damage induced by etoposide treatment whereas FK ESCs could not.

(D) DNA integrity assessment of WT, FK, FK-340, and FK-Filia ESCs by comet assay showed persistent DNA breaks in FK ESCs.

(E) Rad51 was recruited to DSB sites in WT, FK-340, and FK-Filia ESCs upon DNA damage. However, its recruitment was compromised in FK ESCs. Squares indicate examples of γH2AX^{+} Rad51^{-} foci. 50 cells were examined in each sample.

Data are represented as mean ± SEM. Scale bars, 10 μm. ***$p < 0.001$. See also Figure S3.

Of interest, Filia expression exhibited a similar oscillation pattern as ATM activation in WT ESCs (Figure 2C). To further explore the relationships among Filia, ATM, and Chk2, we inhibited ATM kinase activity using a specific ATM inhibitor, KU55933, and examined Filia expression and Chk2 activation. Inhibition of ATM activity did not affect Filia expression but impaired Chk2 activation (Figure S3H). These data suggest that Filia functions upstream of ATM in DDR.

Filia Regulates Cell Cycle Checkpoints and DNA Damage Repair

Cell-cycle checkpoint and DNA repair rely on DDR signaling (Branzei and Foiani, 2008). For instance, Chk1 is required for initiation of the G2/M checkpoint in mESCs (Liu et al., 2000), and Chk2 is required for the maintenance of G2/M arrest (Hirao et al., 2000). ATM is critical for both G2/M checkpoint and DNA repair in ESCs (Momcilović et al., 2009; Yamamoto et al., 2012). Cell-cycle analysis revealed that the S and G2/M checkpoints were impaired in FK ESCs, which could be rescued by Filia (Figures 3A and 3B). Intriguingly, expression of Filia340 failed to restore cell-cycle checkpoints despite its ability to rescue DDR signaling (Figures 3A and 3B). Therefore, cell-cycle checkpoint defects in FK ESCs were not simply a consequence of DDR signaling failure. Rather, Filia itself participated in the regulation of cell-cycle checkpoints, and this function required its C terminus (aas 341–440).

ATM activation is essential for DNA damage repair (Yamamoto et al., 2012). To investigate whether Filia loss impairs DNA damage repair, we performed a comet assay to evaluate the repair competence in WT, FK, FK-Filia, and FK-340 rescue ESCs. ESCs were treated with etoposide, and the extent of DNA damage was evaluated after 0 hr, 6 hr and 12 hr of recovery. Notably, WT, FK-Filia, and FK-340 cells displayed a similar competence in DNA damage repair, whereas FK cells exhibited an impairment in damage repair (Figure 3C). Although Filia340 was less efficient than Filia at 6 hr, this difference disappeared at 12 hr post-recovery (Figure 3C). Consistently, FK-Filia and FK-340 ESCs showed similarly mild DNA damage compared with WT cells (Figure 3D), supporting the notion that re-introduction of Filia or Filia340 into FK ESCs is sufficient to restore DNA damage repair. Rad51 is a key protein involved in HR-mediated DSB repair. In WT as well as in FK-Filia and FK-340 rescue ESCs treated with etoposide, most γH2AX foci were co-localized with the Rad51 foci when examined 12 hr post-damage (Figure 3E). However, in FK

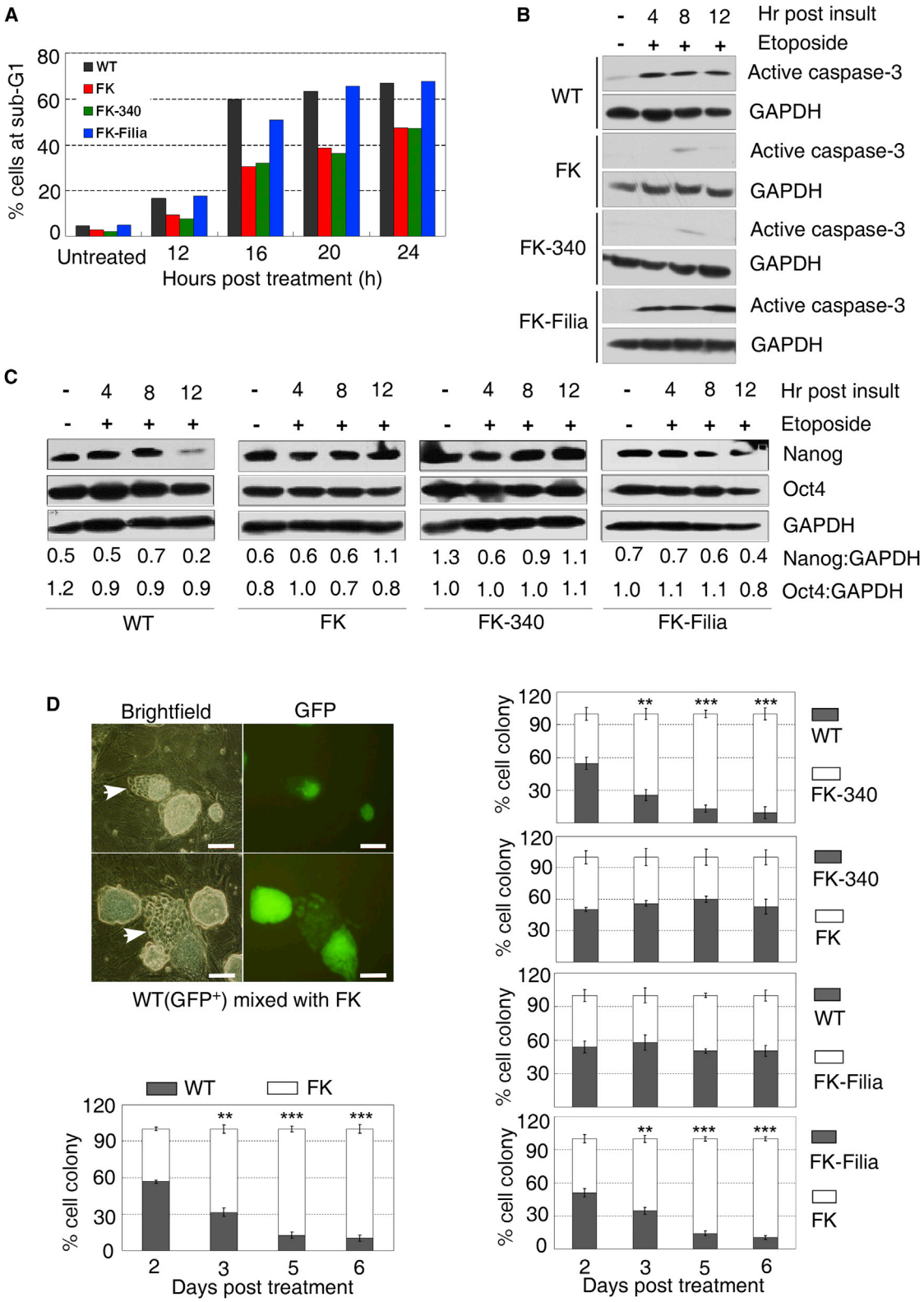

Figure 4. Filia Is Required for ESCs to Undergo Differentiation and Apoptosis in Response to DNA Damage

(A) FACS analysis showed that a higher proportion of WT and FK-Filia ESCs were at sub-G1 phase, indicative of cell death after etoposide treatment.

(B) WT and FK-Filia ESCs expressed more active caspase-3 than FK and FK-340 ESCs in response to etoposide treatment.

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ESCs, the number and size of Rad51 foci decreased, and there were a significant number of γH2AX foci lacking co-localized Rad51 (Figure 3E). Notably, the recruitment of active DNA-PKcs to DNA damage sites, a marker of NHEJ-mediated DSB repair (Davis et al., 2014), was not affected by Filia loss (Figure S3I). This suggests that Filia depletion preferentially impairs HR-mediated DSB repair. Rad51 protein was expressed at comparable levels in WT, FK, FK-340 or FK-Filia ESCs (Figure S3J), implying that Filia facilitates the efficient recruitment of Rad51 to the damage sites. It is intriguing that Filia340-rescued cells are capable of repairing DNA damage despite the lack of cell-cycle checkpoints. This suggests that cell-cycle arrest is not a prerequisite for DNA damage repair and that the two processes are regulated independently.

Filia Regulates Differentiation and Apoptosis of ESCs in Response to DNA Damage

Elimination of cells with irreparable DNA damage is the last and most critical safeguarding event in DDR. Stem cells display hypersensitivity to DNA damage (Dumitru et al., 2012; Liu et al., 2013) and are primed to undergo rapid differentiation and apoptosis to ensure genome stability (Inomata et al., 2009; Lin et al., 2005). FK ESCs did not encounter culture crisis despite accumulated spontaneous DNA damage, suggesting that hypersensitivity to DNA damage is impaired in these cells. To test this hypothesis, we treated the ESCs with etoposide and investigated the dynamics of differentiation and apoptosis. Sub-G1 apoptotic cell analysis revealed that FK ESCs were more resistant to cell death than WT ESCs (Figure 4A). Consistently, there was a decrease of cleaved caspase-3, a critical executioner and marker of apoptosis, in FK compared with WT ESCs (Figure 4B). This phenotype was rescued by Filia but not Filia340 (Figures 4A and 4B). This result indicates that the C terminus of Filia (aas 341–440) was necessary for this function. ESC differentiation is triggered by transactivation of p53 that binds to the enhancer region of *Nanog* to suppress its expression (Li et al., 2012; Lin et al., 2005). Consistently, Nanog, but not Oct4, displayed significant downregulation 12 hr after damage in WT and FK-Filia ESCs but not in FK or FK-340 cells (Figure 4C).

To more comprehensively evaluate the functional outcome of Filia loss on cell survival in response to DNA damage, we performed a clonal competition assay in which same numbers of two types of ESCs were mixed, exposed to DNA damage, and co-cultured for 6 days. To distinguish the two mixed cell types, one was labeled with GFP expression. Compared with WT ESCs, FK cells showed a higher survival rate (Figure 4D). Furthermore, WT, but not FK ESCs, displayed a flattened morphology indicative of ESC differentiation (Figure 4D). Consistently, expression of Filia, but not Filia340, in FK ESCs restored their hypersensitivity to DNA damage (Figure 4D). Therefore, Filia plays a critical role in ensuring ESCs' hypersensitivity to DNA damage, and this depends on its C terminus.

Phosphorylation of Serine 349 Is Required for Filia Function in DNA Damage Repair

Our data suggest that Filia, akin to p53 in somatic cells, functions in two opposite aspects of DDR in ESCs. It is required for DNA repair, which enables cells to survive the damage. It is also essential for damaged cells to undergo differentiation and apoptosis. Phosphorylation often correlates with a change in protein functions. There is evidence to suggest that the S349 residue on Filia is subjected to phosphorylation in response to DNA damage (Pines et al., 2011). Therefore, we investigated whether S349 was indeed phosphorylated and whether this modification played a role in modulating Filia's functions. Accordingly, we mutated S349 into alanine (S349A) that can no longer be phosphorylated and stably expressed this mutant in FK ESCs (FK-S349A) (Figure S4A). FiliaS349A protein rescued the observed defects in DDR signaling (Figure S4B) and intra-S and G2/M cell-cycle checkpoints in FK ESCs (Figures S4C and S4D). However, it failed to restore DNA repair, as demonstrated by the comet assay under normal and etoposide-treated conditions (Figures 5A and 5B). Consistently, FiliaS349A failed to rescue Rad51 recruitment to damage sites (Figure S4E). As a result, FK-S349A ESCs were more sensitive to DNA damage and prone to undergo apoptosis compared with WT ESCs (Figures 5C–5E). Consequently, these cells could not be maintained in culture for more than 12 passages. To further validate the phosphorylation of S349, we generated a polyclonal antibody that specifically recognizes the phosphorylated Filia at S349 (p-Filia(S349)). Immunoblotting revealed a specific, ~70-kDa band that displayed increasing intensity in response to DNA damage in WT but not FK-S349A ESCs (Figure 5F). Together, these data indicate that the S349 residue of Filia is phosphorylated in response to DNA damage and that this modification is essential for Filia's function in DNA damage repair. Moreover, S349 is not a substrate of ATM; suppressing ATM kinase activity with KU55933 does not affect S349 phosphorylation (Figure S3H).

To further explore the biological significance of S349 phosphorylation, we substituted serine with aspartic acid to mimic its phosphorylation and stably expressed FiliaS349D in FK ESCs (FK-S349D rescue cells; Figure S4A). FiliaS349D restored DDR signaling (Figure S4B) and cell-cycle checkpoints (Figures S4C and S4D) but failed to rescue the damage repair (Figures 5A and 5B; Figure S4E). Surprisingly, S349D severely impaired Filia's ability in regulating apoptosis, which correlates with a hyper-toleration of FK-S349D cells to DNA damage compared with either WT or FK ESCs (Figures 5D, 5G, and 5H). Together, these data support the notion that S349 phosphorylation is required for DNA repair, whereas the non-phosphorylation status might be critical for Filia's pro-apoptotic function.

Filia Dynamically Translocates to Different Sub-Cellular Sites in Response to DNA Damage

We next examined Filia's sub-cellular localization under normal and DNA damage conditions. 3xFLAG-tagged Filia, Filia340,

(C) Nanog was downregulated in WT and FK-Filia but not in FK or FK-340 ESCs after DNA damage.
(D) Clonal competition assay revealed that FK ESCs were less sensitive to DNA damage than WT ESCs. Re-expression of Filia, but not Filia340, restored their sensitivity to DNA damage. Shown are representative images of mixtures of WT (GFP^+) and FK ESC clones. Note that WT ESC clones (white arrow) initiated differentiation. Data are represented as mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$.

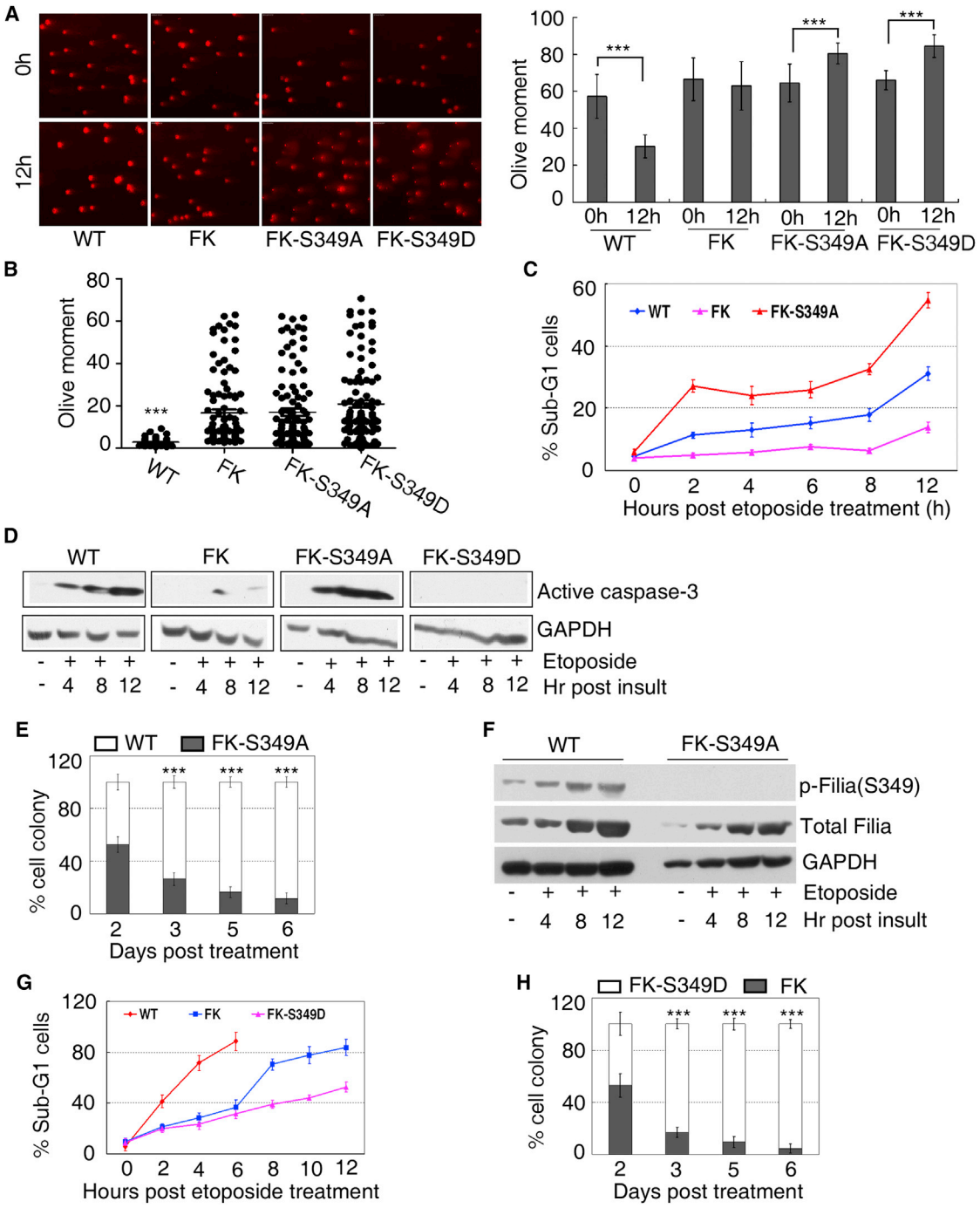

Figure 5. S349 Is Phosphorylated to Modulate Filia's Role in DNA Damage Repair

(A) After etoposide treatment, WT ESCs were more efficient to repair DNA damage than FK, FK-S349A, and FK-S349D ESCs.

(B) Consistently, untreated FK, FK-S349A, and FK-S349D ESCs had severe DNA damage compared with WT ESCs.

(C) Higher proportion of FK-S349A ESCs were dead compared with WT and FK ESCs after etoposide treatment.

(D) Consistently, FK-S349A ESCs expressed more active caspase-3 than WT, FK, and FK-S349D ESCs.

(E) A clonal competition assay confirmed that FK-S349A ESCs had superior sensitivity to DNA damage than WT cells.

(F) Immunoblotting with antibody specifically recognizing phosphorylated S349 in Filia validated this phosphorylation modification. Note that the phosphorylation level was upregulated by DNA damage.

(G) Sub-G1 analysis indicated that FK-S349D ESCs were more resistant to apoptosis.

(H) A clonal competition analysis further confirmed the higher tolerance of FK-S349D ESCs to DNA damage than FK ESCs.

Data are represented as mean ± SEM. ***$p < 0.001$. See also Figures S3 and S4.

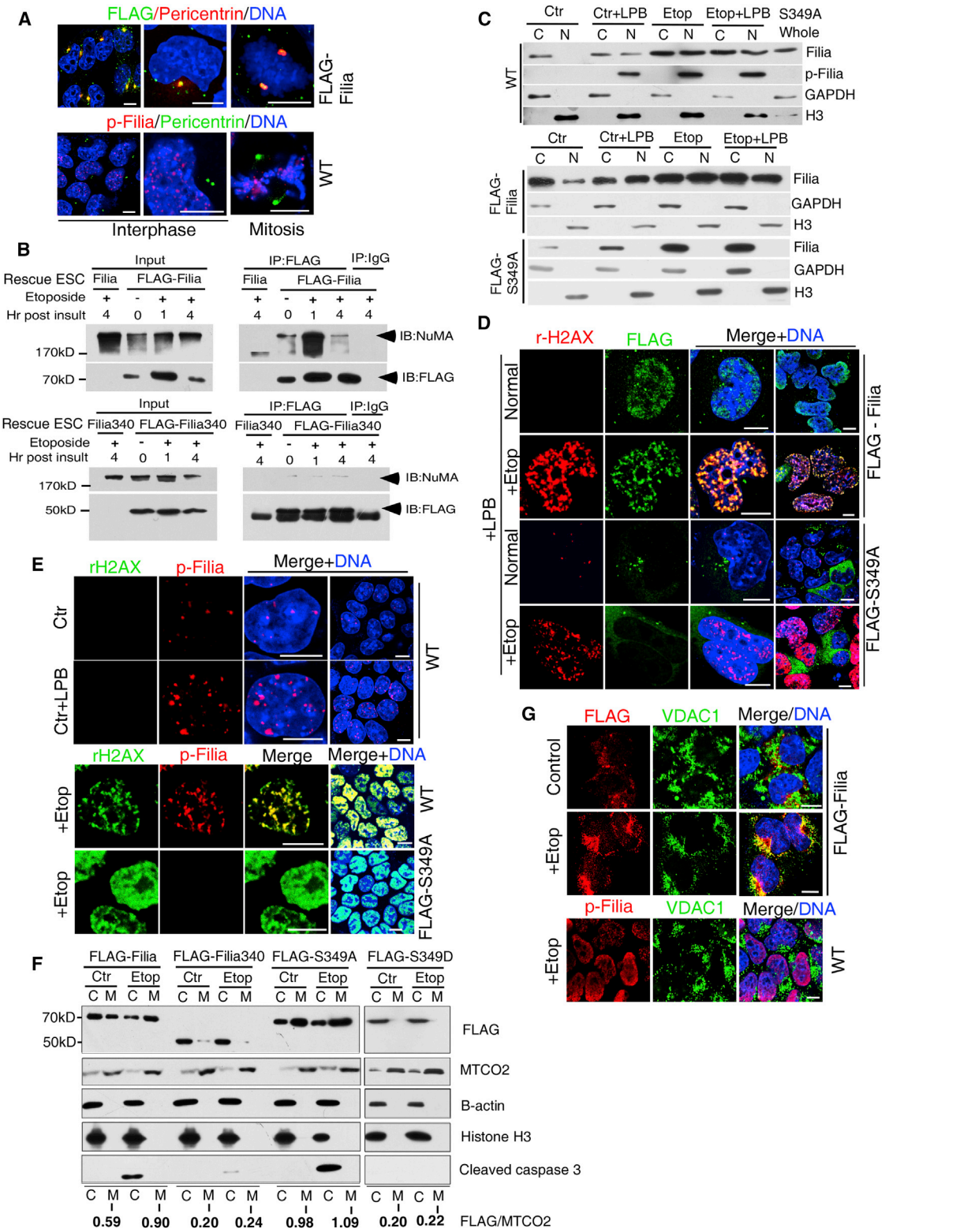

Figure 6. Filia Constitutively Localizes on Centrosomes and DNA Damage Stimulates Its Translocation to Damage Sites or Mitochondria

(A) Constitutive localization of Filia, but not p-Filia(S349), on centrosomes labeled by pericentrin.

(B) Immunoprecipitation (IP) combined with immunoblotting (IB) confirmed the interaction of Filia or Filia340 with Numa.

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FiliaS349A, or FiliaS349D were stably expressed in FK ESCs. Immunostaining revealed that Filia is primarily located in cytoplasm regardless of cell-cycle or genotoxic damage (Figure S5A). Centrosomes are considered as command centers for cellular control and are known to integrate cell-cycle regulation and DNA repair (Doxsey et al., 2005; Löffler et al., 2006; Shimada and Komatsu, 2009). To examine whether Filia is localized on centrosomes, we extracted free cytosolic Filia and co-stained FLAG-tagged Filia with pericentrin and γ-tubulin, two integral components of pericentriolar material. Filia accumulated on centrosomes at interphase and mitotic phase (Figure 6A; Figure S5B) under normal conditions. DNA damage did not enhance the centrosomal accumulation of Filia (Figure S5B), suggesting that this localization was constitutive. Centrosomal localization was also observed for Filia340, FiliaS349A, and FiliaS349D (Figure S5C). However, p-Filia(S349) did not localize to the centrosome (Figure 6A). Interestingly, Chk2 resides on centrosomes of mESCs (Hong and Stambrook, 2004). p-ATM was also detected on centrosomes of WT and rescue ESCs but not FK cells (Figure S5D). These findings suggest that centrosome-localized Filia may play a role in regulating ATM, Chk2, and cell-cycle checkpoints.

The constitutive centrosomal localization of Filia implied a role in regulating centrosome integrity. Centrosomes in FK ESCs were abnormal (Figure 1J). This could be a consequence of DNA damage (Bourke et al., 2007; Löffler et al., 2013). To exclude this possibility, we examined the centrosome integrity of FK-Filia, FK-Filia340, FK-S349A, and FK-S349D rescue ESCs. Notably, all examined ESCs displayed grossly normal centrosomes and spindle assemblies (Figure S5E), despite the fact that sustained DNA damage was observed in FK-S349A and FK-S349D ESCs (Figure 5B). This observation excluded the causal relationship of DNA damage and centrosome defects observed in FK ESCs. Rather, it suggests that Filia itself plays a direct role in maintaining centrosome integrity. Indeed, co-immunoprecipitation combined with mass spectrometry identified Numa, a critical regulator of spindle pore integrity (Silk et al., 2009; Zeng, 2000), as an interacting protein of Filia on the centrosome (Figure 6B). The C terminus of Filia (aas 341–440) contributes to this interaction because there was an impairment of interaction between Numa and Filia340 compared with full-length Filia (Figure 6B).

In addition to the cytoplasmic distribution, there was a small amount of Filia in nuclei, as determined by immunostaining and nucleus fractionation (Figure S5A; Figure 6C). To confirm the presence of nuclear Filia, we treated the WT and FLAG-Filia rescue ESCs with leptomycin B (LPB) to inhibit nuclear protein export (Alpatov et al., 2014; Tamanini et al., 1999). Nuclear localization of Filia was clearly visible after LPB treatment (Figure 6D). Under normal conditions, Filia was diffused in the nucleus. DNA damage evoked an increase in both the abundance of nuclear Filia and its translocation to the damage sites, as labeled by γH2AX (Figures 6C and 6D). Intriguingly, S-to-A mutation (FLAG-S349A cells) prevented the entry of Filia into nuclei regardless of DNA damage (Figures 6C and 6D), indicating that Filia S349 phosphorylation is required for its nuclear localization. Consistently, p-Filia(S349) was exclusively stained for nuclei of WT ESCs under normal conditions and co-localized with γH2AX upon DNA damage (Figures 6C and 6E). Filia340 showed a similar nuclear distribution as full Filia (Figures S6A and S6B), which correlates with its ability to restore DNA repair. Intriguingly, FiliaS349D protein exhibited proper nuclear localization (Figures S6A and S6B) despite its inability to repair DNA damage.

We next determined whether Filia translocated to mitochondria upon apoptosis induction. Under normal conditions, a basal level of Filia was detected in mitochondria, as assayed by immunostaining and mitochondrial fractionation. Apoptosis induction with etoposide triggered a robust translocation of Filia into mitochondria (Figures 6F and 6G). Filia localization to mitochondria was compromised in Filia340 and FiliaS349D, whereas it was enhanced in FiliaS349A (Figure 6F; Figure S6C). These results support the notion that localization of Filia in mitochondria is necessary for its apoptosis-promoting role. They also implied that mitochondrial translocation of Filia requires its C terminus and S349 at the non-phosphorylated status. Consistently, p-Filia(S349) was not detected in mitochondria (Figure 6G).

Filia Interacts with PARP1 and Stimulates Its Enzymatic Activity in DDR

To further explore the mechanistic basis by which Filia regulates DDR, we performed co-immunoprecipitation combined with mass spectrometry analysis. We identified PARP1 as one of Filia's interaction proteins (Figure 7A). PARP1 catalyzes the poly(ADP-ribosyl)ation (PARylation) of its target proteins and plays a key role in early DDR (De Vos et al., 2012; Krishnakumar and Kraus, 2010). The interaction between PARP1 and Filia was validated in NIH/3T3 cells ectopically expressing Filia (Figure 7B). The interaction between PARP1 and Filia is regulated, and it was enhanced by DNA damage (Figure 7A). Moreover, the interaction did not require the C terminus of Filia because Filia340 pulled down PARP1 efficiently (Figure 7A). Although these two proteins interact, there was no obvious co-localization between them. PARP1 was predominantly distributed in nuclei (Figure S7A), whereas the majority of Filia

(C) Nucleus fractionation documented the presence of Filia in the cytoplasm (C) and nucleus (N) of WT and FLAG-Filia rescued ESCs. However, FiliaS349A and p-Filia(S349) were exclusively detected in the cytoplasm and nucleus, respectively. LPB as well as etoposide treatment increased the nuclear accumulation of Filia and p-Filia. Whole lysates of FK-S349A ESCs were used as a control. Ctr, control. Etop, etoposide.

(D) Immunostaining revealed the nuclear localization of Filia but not FiliaS349A proteins. Etoposide treatment stimulated the recruitment of Filia to DNA damage sites labeled with γH2AX.

(E) In WT ESCs, p-Filia(S349) was detected in the nucleus under normal and DNA damage conditions. Etoposide treatment increased the accumulation of p-Filia(S349) on DNA damage sites. FK-S349A cells served as a negative control.

(F) Mitochondria fractionation revealed the localization of Filia and FiliaS349A on mitochondria (M) marked by MTCO2. Apoptosis induction by etoposide treatment evoked their accumulation on mitochondria. However, little Filia340 or FiliaS349D protein was detected on mitochondria under normal or DNA damage conditions. C represents the fraction of whole-cell lysates depleted of mitochondria.

(G) Immunostaining confirmed the localization of Filia but not p-Filia(S349) on mitochondria marked with VDAC1.

Scale bars, 10 μm. See also Figures S5 and S6.

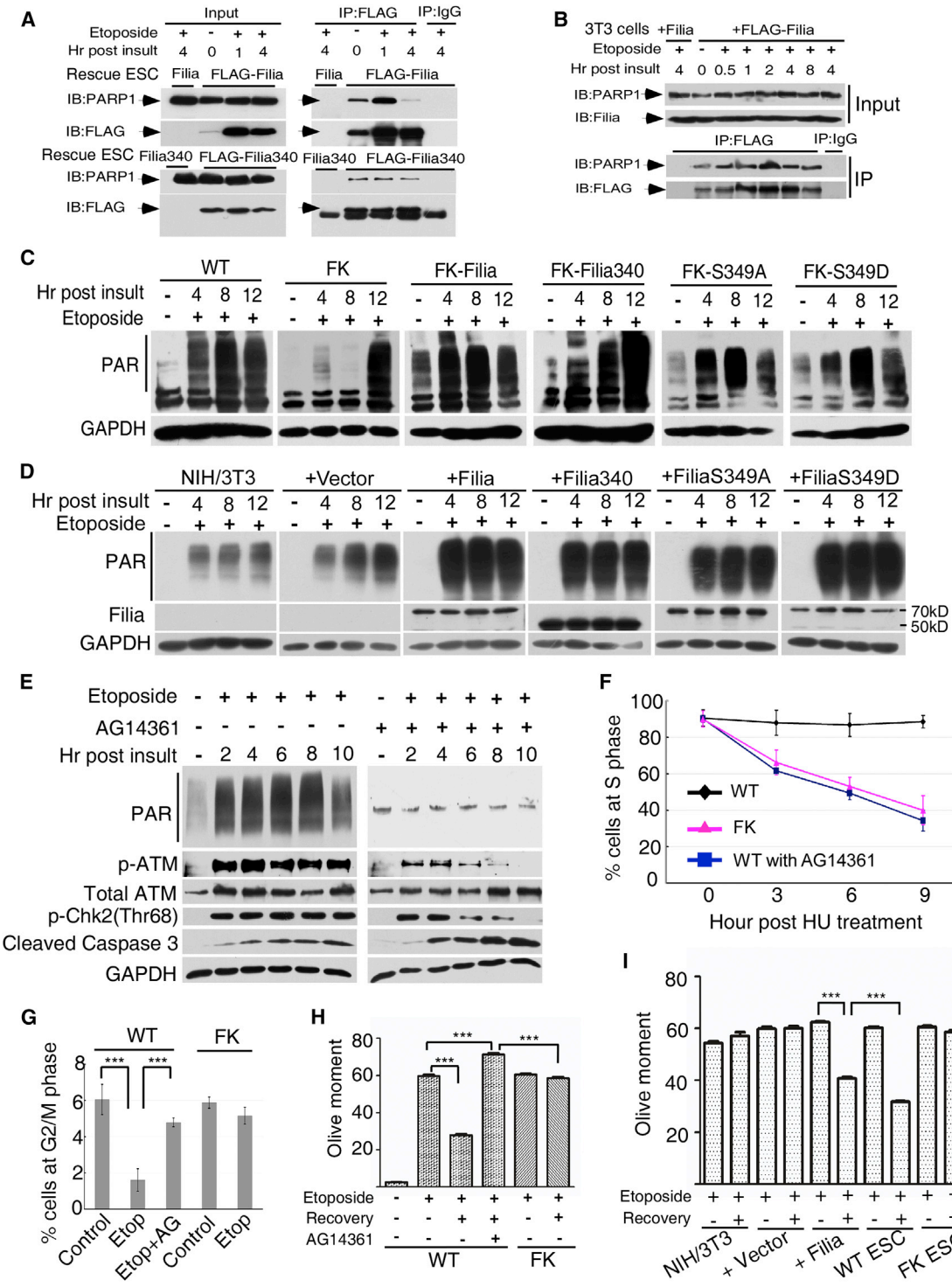

Figure 7. Filia Interacts with PARP1 and Stimulates Its Enzymatic Activity Which Amplify Filia's Roles in DDR

(A) Immunoprecipitation combined with immunoblotting confirmed the interaction of Filia or Filia340 with PARP1 in ESCs under normal or DNA damage conditions.

(B) Filia interacts with PARP1 in NIH/3T3 cells ectopically expressing Filia under normal or DNA damage conditions.

(C) FK ESCs had a much lower PAR level than WT ESCs. However, re-expression of Filia or its variants restored the PAR level.

(D) Similarly, ectopic expression of Filia or its variants in NIH/3T3 cells significantly increased the PAR level in response to DNA damage.

(E) Inhibition of PARP1 activity by AG14361 significantly attenuated ATM and Chk2 activation. Consequently, cells with deficient PARP1 activity were prone to undergo apoptosis.

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was detected in the cytoplasm (Figure S5A). Unlike Filia (Figure 6D), PARP1 nuclear foci were typically not co-localized with γH2AX foci induced by DNA damage (Figure S7A). These observations support the notion that the interaction between Filia and PARP1 is transient.

To determine whether PARP1 is responsible for PARylation in ESCs, we inhibited PARP1 enzymatic activity using a specific inhibitor, AG14361 (Calabrese et al., 2004; Figure S7B). Time course analysis revealed that inhibition of PARP1 completely abolished PARylation (Figure S7C), indicating that PARP1 plays a major role in regulating PARylation in ESCs. We next examined whether Filia regulates PARP1 activity by comparing PAR levels between WT and FK ESCs. In WT ESCs, PAR levels increased after etoposide treatment, whereas significantly less PAR was detected in untreated or etoposide-treated FK cells examined at 4 and 8 hr (Figure 7C). Therefore, Filia is necessary for robust PARP1 activation in response to DNA damage. Intriguingly, PARP1-dependent but Filia-independent PAR was elevated at 12 hr in FK cells (Figure 7C; Figure S7C). The Filia-independent PAR displayed abnormal accumulation at the nucleolus (Figure S7D), which is known as a storage site for PAR in DDR (Mortusewicz et al., 2007). Re-expression of Filia, Filia340, FiliaS349A, or FiliaS349D in FK ESCs efficiently restored PARP1 activity and PAR levels (Figure 7C). Importantly, differentiating ESCs and somatic cells have much less PAR compared with undifferentiated ESCs (Figures S7E and S7F). Ectopic expression of Filia and its variants in NIH/3T3 cells significantly increased PAR levels (Figure 7D). The majority of PAR was detected in nuclei of ESCs expressing Filia, Filia340, and FiliaS349D, whereas PAR was predominantly accumulated in the cytoplasm of FK-S349A rescue cells (Figure S7G). This is consistent with the cytoplasmic distribution of FiliaS349A. Notably, localization of Filia to DNA damage site did not rely on PAR modification (Figure S7H).

To determine whether PARP1 plays a role in mediating Filia function in DDR, we inhibited PARP1 activity using AG14361 and examined its effects on ATM and Chk2 activation, cell-cycle checkpoints, DNA damage repair, and apoptosis. Inhibition of PARP1 significantly attenuated, but did not completely block, ATM and Chk2 activation (Figure 7E). Notably, PARP1 inhibition caused the same extent of defects in cell-cycle checkpoints and DNA damage repair as Filia knockout (Figures 7F–7H). Consistently, ectopic expression of Filia in NIH/3T3 cells not only increased PAR levels (Figure 7D) but also enhanced DNA damage repair in these cells (Figure 7I). However, DNA damage-induced apoptosis was not impaired by PARP1 inhibition (Figure 7E). This suggests that PARP1 activity is not necessary for apoptosis induction. Together, these data support the notion that Filia interacts with PARP1 and activates its enzymatic activity in response to DNA damage, which contributes to the observed roles of Filia in regulating DDR signaling, cell-cycle checkpoints, and DNA damage repair.

DISCUSSION

Compared with somatic cells, PSCs have superior competence and unique strategies to cope with DNA damage to maintain genomic integrity. In addition to using common proteins found in somatic cells with alternative strategies (Dumitru et al., 2012), PSCs possess unique proteins to safeguard their genome integrity (Zalzman et al., 2010). However, little is known regarding the PSC-specific factors in regulating genomic stability. Here we identify an ESC-specific protein, Filia, as a powerful regulator of genomic stability. Through its coordinated cytoplasmic and nuclear functions, Filia regulates centrosome integrity and DDR at multiple levels. These include DDR signal transduction, cell-cycle checkpoints, DNA damage repair, and apoptosis. Therefore, Filia depletion not only causes robust genomic instability but also impedes the elimination of damaged cells by ESC differentiation or apoptosis. This, in turn, increases the risk of transformation and tumorigenesis in ESCs. In somatic cells, p53 plays similar dual regulatory roles in DDR (Green and Kroemer, 2009). However, these well established roles of p53 are not evident in mESCs (Zhao and Xu, 2010). We hypothesize that PSCs utilize specific factors such as Filia to safeguard their genome integrity. Of note, reprogramming somatic cells into induced PSCs (iPSCs) is characterized by large variation in *Filia* (*Ecat1*) expression (Aoi et al., 2008; Kaji et al., 2009; Takahashi and Yamanaka, 2006). This suggests that *Filia* expression is a potential molecular marker that correlates with iPSC quality.

Filia carries out its multiple functions through different mechanisms. On one hand, Filia is dynamically translocated to centrosomes, DNA damage sites, and mitochondria to execute its regulation on DDR independent of PARP1. On the other hand, Filia physically interacts with PARP1 and stimulates PARP1's enzymatic activity to regulate DDR. PARP1 plays a key role in regulating DDR and genomic integrity (Krishnakumar and Kraus, 2010; Luo and Kraus, 2012). For instance, PARP1- or PAR-deficient cells are compromised in ATM activation, DNA damage signaling, cell-cycle checkpoints, and DNA repair (Aguilar-Quesada et al., 2007; Haince et al., 2007; Min et al., 2013). Knockout mice for *Parp1* or *Parp2* are hypersensitive to DNA-damaging agents and display increased genomic instability after genotoxic stress (Hassa et al., 2006). More than 100 PARylation targets were identified in DDR, including PARP1 itself, histones, CDK2, kinases, and damage repair proteins (Jungmichel et al., 2013). Filia is uniquely expressed in ESCs, which correlates with high PAR levels and a superior ability to maintain genomic stability in these cells. Therefore, these findings support a model in which activation of PARP1 by Filia contributes to the observed phenotypes in ATM and Chk2 activation, cell-cycle checkpoints, and DNA damage repair in FK ESCs.

Filia and PARP1 do not apparently co-localize. In addition, FiliaS349A is able to stimulate PARP1 activity despite its restrictive cytoplasmic distribution. These findings suggest

(F–H) Inhibition of PARP1 activity in ESCs abolished S phase (F) and G2/M (G) cell-cycle checkpoints and compromised DNA damage repair (H).
(I) Ectopic expression of Filia in NIH/3T3 cells significantly enhanced their DNA damage repair ability. NIH/3T3 cells, NIH/3T3 cells transfected with vectors, WT ESCs, and FK ESCs were used as controls.
In (H) and (I), cells were recovered for 12 hr.
Data are represented as mean ± SEM. ***$p < 0.001$. See also Figure S7.

that the dynamic interaction between PARP1 and Filia and activation of PARP1 by Filia occur in both the cytoplasm and nuclei. PAR regulates the protein's sub-cellular redistribution, and this provides a possible explanation for the presence of PAR and p-ATM in the nuclei of FiliaS349A-rescued ESCs.

Compared with Filia knockout, PARP1 inhibition caused a less obvious defect in ATM and Chk2 activation in response to DNA damage. Filia340 failed to rescue the defects in cell-cycle checkpoints despite its ability to activate PARP1. These findings suggest that Filia also regulates ATM-Chk2 activation and cell-cycle checkpoints in a PARP1-independent manner. ATM activation exhibits two phases, and the first phase is not overtly affected by Filia knockout. This might be due to the presence of basal levels of PAR in Filia knockout cells, which accounts for the first phase of ATM activation. Centrosomes are known to integrate many regulatory factors that control cell-cycle progression and DDR. Cell-cycle regulators such as the Cdk-cyclin complex (Bailly et al., 1992; Hinchcliffe et al., 1999), Chk1 (Krämer et al., 2004; Zhang et al., 2007), and Chk2 (Golan et al., 2010; Hong and Stambrook, 2004; Zhang et al., 2007) are present on centrosomes. Furthermore, DDR regulators such as ATM, ATR, and DNA-PK have also been shown to reside on centrosomes (Zhang et al., 2007). Consistently, we observed the localization of p-ATM on centrosomes of ESCs. However, p-ATM was absent from centrosomes when Filia was depleted. Re-expression of Filia340, FiliaS349A, or FiliaS349D, all of which localize on centrosomes, restored a second wave of ATM activation. Therefore, the centrosomally localized Filia may coordinate the control of DDR signaling, cell-cycle checkpoints, and centrosome integrity.

FiliaS349A- or FiliaS349D-rescued ESCs fail to repair DNA damage despite the PARP1 activity and PAR levels being normal. This is consistent with the idea that Filia also regulates DNA damage repair in a PARP1-independent manner. The phosphorylation of S349 at the C terminus is necessary for Filia's nuclear localization. However, without the C terminus, Filia340 is localized to nuclei (Figure S6B). Notably, bioinformatics analysis indicates that Filia340 (aas 1–340) contains a potential nuclear localization signal (NLS). Therefore, it is possible that the phosphorylation of S349 regulates NLS function. Specifically, non-phosphorylated S349 suppresses NLS function, whereas phosphorylation of S349 or simply removing the C terminus activates NLS function. Upon LPB treatment, nuclear Filia is diffusive in the absence of DNA damage. DNA damage triggers the translocation of Filia to DNA damage sites, indicating that Filia's entry into nuclei and its translocation to DNA damage sites are regulated separately. PARylation plays a critical role in recruiting DNA repair proteins to damage sites (Krishnakumar and Kraus, 2010; Tallis et al., 2014). However, localization of Filia into DNA damage sites is regulated neither by PARylation nor by interaction with PARP1.

In summary, our data demonstrate that Filia functions as the first of its kind ESC-specific regulator of genome integrity. These data support the notion that ESCs employ distinct mechanisms and utilize specific factors, such as Filia, to safeguard their genomic integrity. They also suggest that the Filia expression level is a potential biomarker for the quality of iPSCs with regard to genomic stability and transformation potential.

EXPERIMENTAL PROCEDURES

Derivation and Culture of Mouse ESCs

All experimental procedures and animal care were performed according to the protocols approved by the Ethics Committee of the Kunming Institute of Zoology, Chinese Academy of Sciences. MEF preparation, ESC derivation, and culture were performed in standard ways (Evans and Kaufman, 1981). ESC genotyping was performed as described previously (Zheng and Dean, 2009).

Cytogenetic Analysis and T-FISH

Metaphase chromosome spreads were prepared and stained with Giemsa solution or DAPI. For telomere fluorescent in situ hybridization (T-FISH), metaphase spreads were prepared. DNA was denatured and hybridized with peptide-nucleic acid (PNA) telomere probes (Tel G-Alexa 488, PANAGENE, F1010-5) (Lansdorp et al., 1996). DNA was counterstained with 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI).

Immunoblotting and Immunofluorescent Staining

Immunoblotting and immunofluorescent staining were performed according to standard procedures (Närvä et al., 2012). The primary antibodies are listed in Table S1. Rabbit polyclonal antibodies against Filia and p-Filia(S349) were generated by Abmart.

Cell-Cycle Checkpoint Analysis

ESCs were treated with hydroxyl urea to induce replication stress, and S-phase arrest was evaluated by 5-ethynyl-2'-deoxyuridine (EdU) pulse-chase incorporation assay (Buck et al., 2008). The G2/M checkpoint was examined by standard methods after treating ESCs with etoposide (Greer Card et al., 2010).

Alkaline Comet Assay and Clonal Competition Assay

An alkaline comet assay was performed according to the standard procedure (Tice et al., 2000). Comets were analyzed using CASP comet assay analysis software (Andor Technology), and 100 cells were calculated in each sample. The same numbers of two types of ESCs, of which one was labeled with GFP expression, were mixed. The mixed ESCs were subjected to the same genotoxic stress and co-culture. Colony growth was monitored daily, and 200 clones were examined at each time point.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2015.03.017.

ACKNOWLEDGMENTS

We thank Dr. Jurrien Dean at the NIH for providing *Filia* mutant mice; Wei-jian Sang, Rui Bi, and Zhengyuan Xie for technical support in making lentiviral expression vectors; the Protein Mass Spectrometry Center at the Shanghai Institute of Materia Medica, CAS, for performing the mass spectrometry analysis; and Dr. Jurrien Dean at the NIH and Dr. Jiali Li at the Kunming Institute of Zoology, CAS, for carefully reading and editing the paper. This work was supported by the Chinese NSFC grant 31171424 (to P.Z.), by CAS Strategic Priority Research Program Grant XDA01010203, and by a talent grant from Yunnan Province. R.Z. is supported by NIH/NCI Grant R01CA160331 and Cancer Center Core Grant CA010815.

Received: September 3, 2014
Revised: February 16, 2015
Accepted: March 22, 2015
Published: April 30, 2015

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